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The induction of autoimmune hepatitis in the human leucocyte antigen-DR4 non-obese diabetic mice autoimmune hepatitis mouse model

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Summary

Autoimmune hepatitis (AIH) is a chronic liver disease characterized by progressive inflammation, female preponderance and seropositivity for autoantibodies such as anti-smooth muscle actin and/or anti-nuclear, anti-liver kidney microsomal type 1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1) in more than 80% of cases. AIH is linked strongly to several major histocompatibility complex (MHC) alleles, including human leucocyte antigen (HLA)-DR3, -DR7 and -DR13. HLA-DR4 has the second strongest association with adult AIH, after HLA-DR3. We investigated the role of HLA-DR4 in the development of AIH by immunization of HLA-DR4 (DR4) transgenic non-obese diabetic (NOD) mice with DNA coding for human CYP2D6/FTCD fusion autoantigen. Immunization of DR4 mice leads to sustained mild liver injury, as assessed biochemically by elevated alanine aminotransferase, histologically by interface hepatitis, plasma cell infiltration and mild fibrosis and immunologically by the development of anti-LKM1/anti-LC1 antibodies. In addition, livers from DR4 mice had fewer regulatory T cells (T_{regs}), which had decreased programmed death (PD)-1 expression. Splenic T_{regs} from these mice also showed impaired inhibitory capacity. Furthermore, DR4 expression enhanced the activation status of $CD8^+$ T cells, macrophages and dendritic cells in naive DR4 mice compared to naive wild-type (WT) NOD mice. Our results demonstrate that HLA-DR4 is a susceptibility factor for the development of AIH. Impaired suppressive function of T_{regs} and reduced PD-1 expression may result in spontaneous activation of key immune cell subsets, such as antigen-presenting cells and $CD8^+$ T effectors, facilitating the induction of AIH and persistent liver damage.

Keywords: autoantigen, transgenic-HLA-mouse model, T_{regs}

Introduction

Autoimmune hepatitis (AIH) is a chronic progressive autoimmune disease characterized by hepatocyte inflammation, leading to fibrosis and cirrhosis in many of those affected. AIH affects more females in all age groups. At diagnosis, liver enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elevated. Autoantibodies such as anti-smooth muscle actin (anti-SMA), anti-nuclear (ANA), anti-liver–kidney microsomal type 1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1) are present in more than 80% of patients, in addition to hypergammaglobulinaemia [1–4]. Whereas anti-SMA and ANA antigens are not liver-specific, the target antigens for anti-LKM1 and/or anti-LC1 autoantibodies have been defined as liver proteins: cytochrome P4502D6 (CYP2D6) [5] and formiminotransferase cyclodeaminase (FTCD), respectively. Histological features such as interface hepatitis and plasma cell infiltration are characteristic of AIH [3]. The aetiology of AIH remains poorly understood, which has hindered the identification of more specific treatment to replace the current mainstay of therapy, immune suppression with corticosteroids and/or azathioprine. The therapy is usually lifelong, resulting in undesirable corticosteroid-associated side-effects, including obesity and osteoporosis [6].

Previous studies have demonstrated that immunization of mice with liver autoantigens can induce liver pathology similar to that seen in human AIH, although these models are limited as they lack expression of certain human components [7–10]. We and others have shown that human leucocyte antigen (HLA) alleles such as HLA-DR3, -DR4, -DR7 and -DR13 are associated with AIH [11–15]. We hypothesized that in the presence of a HLA allele known to predispose to AIH, and an autoimmune prone genetic background, the mouse model of human AIH could be improved. To this end, we recently established a novel murine model of AIH by using an autoimmune prone strain of non-obese diabetic (NOD) mouse that also carries the human HLA-DR3 gene [16]. Chronic severe liver damage is induced in these mice after immunization with DNA encoding CYP2D6/FTCD, confirming the important role of DR3 in the development of AIH. Due to the fact that DR4 is either co-expressed with DR3 or another HLA-DR molecule in the same individual, it is difficult to study the independent role of DR4 in human AIH or other autoimmune disorders associated with DR4. Thus, the role of HLA-DR4 remains unclear in AIH. Genetic association studies suggest that after DR3, HLA-DR4 is the second most prevalent disease risk gene in adult patients with AIH [13,17,18].

In this study, we investigated the role of DR4 in the induction of AIH and the effect of DR4 expression on the phenotype and function of immune cells. We generated a human DR4 transgenic NOD mouse, as the NOD genetic background is known to be prone to autoimmunity [19–21]. Immunization of DR4 mice with human liver autoantigens CYP2D6/FTCD resulted in liver injury similar to human AIH, with elevated ALT levels, interface hepatitis, some degree of fibrosis, plasma cell infiltration and the development of anti-LKM1/anti-LC1 autoantibodies, and predominant T helper type 1 (Th1)/Th17 T cell responses. Furthermore, immunized DR4 mice showed a reduced frequency of $CD4^+$forkead box protein 3 $(FoxP3)^+$ T regulatory cells (T_{regs}) in the liver, compared to immunized wild-type (WT, without DR4 gene expression) NOD mice. Interestingly, non-immunized naive DR4 mice also showed dysfunctional T_{regs} with reduced suppressive capacity and lower programmed death 1 (PD-1) expression, activated T cells, macrophages and dendritic cells in the liver. Our study revealed that HLA-DR4 expression enabled the activation of immune system, probably favouring autoimmunity, supporting the notion that HLA-DR4 predisposes to AIH in patients.

Materials and methods

Animals

WT NOD mice were obtained originally from the Jackson Laboratories (Bar Harbor, ME, USA) and maintained at the Yale animal facility for nearly 3 decades. HLA-DR4 transgenic NOD mice were generated by back-crossing HLA-DR4 C57BL/10 mice [22] to the NOD background for > 10 generations. All the mice used in this study were 6–8 weeks old at the time of immunization and housed in specific pathogen-free (SPF) conditions with autoclaved food and bedding in individually ventilated filter cages. All the studies were approved by the Institutional Animal Care and Use Committee of Yale University.

Reagents

Generation of CYP2D6/FTCD DNA construct has been reported previously [7]. The plasmid DNA was transformed to *Escherichia coli* competent cells and purified with the endotoxin free plasmid purification kit (Qiagen, Valencia, CA, USA) after overnight culture. Cytosine–phosphate–guanosine (CpG) ODN (2395, type C) was synthesized by Keck Facility at Yale University. An alanine aminotransferase (ALT) test kit was purchased from Cayman Chemical (Ann Arbor, MI, USA) and serum ALT levels were determined according to the manufacturer's instructions. All the monoclonal antibodies used in this study were purchased from BioLegend (San Diego, CA, USA) or eBioscience (San Diego, CA, USA).

Immunization and liver histology

CYP2D6/FTCD plasmid DNA (100 μg/mouse), together with CpG ODN 2395 as adjuvant (75 μg/mouse), was mixed in phosphate-buffered saline (PBS). HLA-DR4 NOD (DR4) and WT NOD mice were immunized with 100 μl of the antigen mix by intraperitoneal injection. A separate group of DR4 and WT NOD mice were injected with CpG ODN alone as controls. The mice were boosted only once, a month after primary immunization, and bled monthly to test the ALT levels to evaluate liver injury. The experiment was terminated 4 and 7 months after primary immunization to study the short- and long-term effects of immunization on induction of AIH. A piece of liver tissue was fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin and assessed blindly, using the Ishak modified (mISHAK) histology activity scoring system [23] and METAVIR score [24] to evaluate necro-inflammation and fibrosis, respectively. Tissue sections were stained with haematoxylin and eosin (H&E) and Sirius Red to evaluate liver inflammation and fibrosis. All the mice used in this study were non-diabetic.

Liver autoantibody-specific enzyme-linked immunosorbent assay (ELISA)

Autoantibodies against human CYP2D6/FTCD (anti-LKM1/anti-LC1) were measured by ELISA as described previously [25]. Briefly, the fusion protein produced by the pMAL-cR1-CYP2D6-FTCD plasmid (human FTCD and CYP2D6) was purified and used as antigen for coating the

ELISA plate (0·2 μg/well). A serum sample was considered positive if its specific optical density (OD) reading was at least two times higher than the mean OD of the pre-immunized mice sera. Serum samples were diluted from 1 : 10 to 1 : 400.

Serum total IgG ELISA

Mouse IgG Ready-SET-Go kit was purchased from eBioscience (Hatfield, UK) and serum total IgG levels were determined according to the manufacturer's instructions.

Isolation of mononuclear cells and flow cytometry

Prior to tissue harvesting, mouse liver was first perfused with sterile PBS via the portal vein and then weighed, followed by homogenization. Liver mononuclear cells (LMNCs) were harvested at the interface of a 40 and 70% Percoll gradient (GE Healthcare, Piscataway, NJ, USA) after discontinuous gradient centrifugation. Residual red blood cells (RBC) were lysed with RBC lysis buffer (eBioscience). The LMNCs were then washed twice with PBS. The spleen mononuclear cells (SMNCs) were obtained after homogenization and lysis of RBCs with lysing buffer. The live lymphocytes were first gated according to the forward- and side-scatter parameters. The expression of different surface markers and intracellular cytokines (ICC) in LMNCs or SMNCs was analysed by flow cytometry, as described previously [26].

T cell proliferation assay

Antigen-specific T cell responses were tested by culturing splenocytes (2×10^5/well) in the presence of CpG-ODN 2395 (5 μg/ml) with or without CYP2D6/FTCD plasmid (100 μg/ml) for 4 days. [^{3}H]-thymidine was added during the last 16 h of a 4-day culture. T cell proliferation was evaluated by [^{3}H]-thymidine incorporation in a beta plate counter (Perkin Elmer Wallace, Norton, OH, USA). Antigen non-specific T cell response was assayed stimulating the T cells with monoclonal antibody anti-CD3 (2C11 hybridoma supernatant at a dilution of 1 : 300). All the proliferation assays were performed in triplicate.

Regulatory T cell functional assay

T_{reg} function was evaluated by the suppression of mixed lymphocyte reaction (MLR) assay to allogenic antigen. NOD splenocytes (10^5 cells/well) were stimulated with irradiated C57BL/6 splenocytes (5×10^4 cells/well) in the absence or presence of purified T_{regs} ($CD4^+CD25^+$, $1{\cdot}25 \times 10^4$/well), using the T_{reg} purification kit (StemCell Technology, Vancouver, BC, Canada), from WT or HLA-DR4 spleens. MLR was measured by [^{3}H]-thymidine incorporation at the end of 4-day culture. The suppression of MLR by T_{regs} was calculated by the percentage of inhibition of MLR.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 software. Non-parametric two-way analysis of variance (ANOVA) or (non-parametric) Student's *t*-test was used in most experiments and *P*-values of less than 0·05 were considered significant.

Results

CYP2D6/FTCD immunization induced liver injury in DR4 NOD mice

To test whether HLA-DR4 expression in NOD mouse could promote AIH induction, we immunized DR4 and WT NOD mice with CYP2D6/FTCD plasmid DNA together with adjuvant or adjuvant only (control; $n = 6$–12/group) and monitored non-fasting serum ALT levels monthly. ALT was elevated significantly in DR4 mice from 3 months after immunization with autoantigen and adjuvant (Fig. 1a), compared to the autoantigen-immunized WT NOD mice. Seven months after CYP2D6/FTCD immunization, DR4 mice showed the highest level of ALT compared to the mice in other groups (Fig. 1a). Furthermore, the ALT levels in the CYP2D6/FTCD-immunized DR4 mice were significantly higher, as early as 2 months post-immunization than that in the control (adjuvant) immunized DR4 mice (Fig. 1a). However, this difference was not observed after 4 months (Fig. 1a). The ALT levels of adjuvant control mice, with or without DR4 expression, also showed some increase, suggesting that CpG facilitated inflammation whereas the ALT levels of naive DR4 (horizontal dotted line) and WT NOD (horizontal dashed line) mice remained stable during the 7-month observation period (Fig. 1a). As 4 months post-immunization appeared to be a turning point with the first peak of inflammation and remission thereafter, further studies were focused at this time-point.

DR4 and WT NOD mice develop moderate liver damage and plasma cell infiltration after immunization

Parallel to the ALT levels, the necro-inflammation score of the liver (mISHAK) showed that CYP2D6/FTCD-immunized DR4 mice have more liver damage compared to the WT NOD CYP2D6/FTCD-immunized group (Fig. 1b,c, $n = 6$–9). The naive mice did not show any difference in both groups, whereas CpG (control)-immunized groups showed occasional low-grade immune infiltrates (Fig. 1b). Furthermore, we found mild liver fibrosis in some CYP2D6/FTCD-immunized mice (Supporting information, Fig. S1a,b; $n = 6$/group) up to F2 (METAVIR score), with no significant difference between WT and DR4 mice. However, naive or control immunized mice did not develop any fibrosis (Supporting information, Fig. S1a,b).

One of the striking features of AIH is hepatic plasma cell infiltration [27,28]. CYP2D6/FTCD-immunized DR4 mice

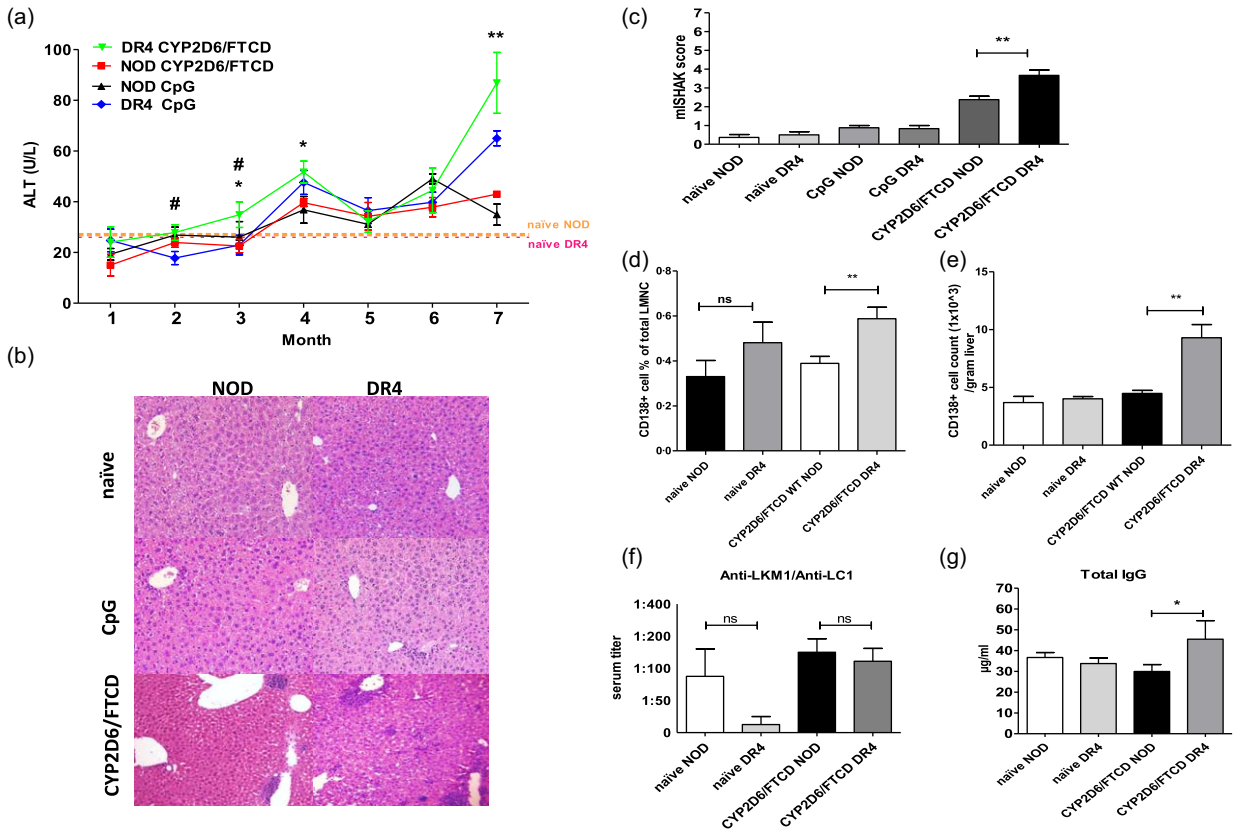

Fig. 1. Liver injury and immune cell infiltration. (a) Serum alanine aminotransferase (ALT) levels: mice were bled monthly (up to 7 months) after immunization ($n = 6$–12/group). (b) Histology: livers from age-matched naive, control-immunized and CYP2D6/FTCD-immunized (4 months post-immunization) were stained with standard haematoxylin and eosin (H&E). Liver histology was viewed under a light microscope (×100) by a blinded investigator. (c) Necro-inflammation within the liver was scored by a blinded investigator ($n = 6$–9). (d,e) Liver $CD138^+$ plasma cells were detected by fluorescence activated cell sorter (FACS) analysis. Liver mononuclear cells (LMNCs) were stained with fluorochrome conjugated anti-CD138 and anti-CD19 antibodies ($n = 3$–8/group). (f) Serum anti-LKM1/anti-LC1 was assessed by enzyme-linked immunosorbent assay (ELISA) ($n = 4$–8/group). (g) Serum total immunoglobulin (Ig)G was investigated by ELISA ($n = 7$–8/group). * or # $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; n.s. = not significant. For ALT graph: *comparison between CYP2D6/FTCD DR4 adjuvant and CYP2D6/FTCD wild-type (WT) non-obese diabetic (NOD) mice; # comparison between CYP2D6/FTCD DR4 adjuvant and control (adjuvants) DR4 mice. Data are from two to three independent experiments and error bars represent the standard error of the mean (s.e.m.) of samples within a group.

had significantly higher frequency and absolute number of $CD138^+$ plasma cells in the liver compared to their CYP2D6/FTCD-immunized WT counterparts (Fig. 1d,e, $n = 3$–8), although $CD138^+$ plasma cells can also be found in some non-immunized naive WT or DR4 mice (Fig. 1d,e, $n = 3$–8/group). It is interesting that naive DR4 mice showed a tendency towards a higher frequency of $CD138^+$ plasma cells in the liver (Fig. 1d); however, the absolute number of these cells was not different from the naive WT NOD mice (Fig. 1e).

DR4 and WT mice developed anti-LKM1/anti-LC1 autoantibody after immunization

To investigate whether CYP2D6/FTCD immunization triggered an autoantigen-specific humoral immune response in WT NOD and DR4 mice, we tested for anti-LKM1/anti-LC1 autoantibodies in the serum. Interestingly, WT NOD and DR4 mice produced similar levels of anti-LKM1/anti-LC1 autoantibodies after immunization (Fig. 1f; $n = 4$–8/group). The naive WT NOD mice were also positive for these autoantibodies, albeit with lower titres compared to immunized mice. It is not clear why naive NOD mice were positive for anti-LKM1/anti-LC1; however, mice with a NOD background are known for inadequate negative selection, which results in spontaneous autoimmunity and autoantibody production [29].

Antigen-immunized DR4 mice develop hypergammaglobulinaemia

Hypergammaglobulinaemia is a hallmark of AIH. We investigated whether the hepatic inflammation in immunized DR4 mice was accompanied by hypergammaglobulinaemia. We measured total serum immunoglobulin (Ig)G levels of the mice in each study group and found that CYP2D6/FTCD-immunized DR4 mice had significantly

higher levels of serum IgG compared to their immunized WT NOD counterparts (Fig. 1g, $n = 8$/group). In contrast, naive DR4 or WT NOD mice were not hypergammaglobulinaemic and showed similar levels of serum IgG (Fig. 1g, $n = 7$/group).

More CD8$^+$ T cells and antigen-presenting cells were present in the liver of immunized DR4 mice

Next, we examined the composition of liver infiltrated immune cells by flow cytometry. There was a significant increase in the number of CD8$^+$ T cells and B220$^+$CD19$^+$ B cells in CYP2D6/FTCD-immunized DR4 mice compared to WT NOD mice (Fig. 2a), whereas immunization-induced CD4$^+$ T cell infiltrates in the liver were similar between DR4 and WT NOD mice (Fig. 2a, $n = 4$–8/group). We further assessed the effector cell markers of liver infiltrated T cells and found a higher frequency of CD4$^+$CD62L$^+$CD44$^+$ T cells in DR4 mice immunized with CYP2D6/FTCD, although it did not reach statistical significance compared to immunized WT NOD mice (Fig. 2b,c, $n = 4$–8). However, a significantly higher frequency of CD62L$^+$CD44$^+$ effector CD8$^+$ T cells was seen in CYP2D6/FTCD-immunized DR4 mice compared to the immunized WT NOD mice (Fig. 2d,e, $n = 4$–8). It is interesting that naive DR4 mice also showed a higher frequency of effector CD8$^+$ T cells in comparison to naive NOD mice (Fig. 2e, $n = 4$–8), although the absolute number of hepatic CD8$^+$ T cells in DR4 mice was similar to naive WT NOD mice (Fig. 2a). We also studied the activation status of CD11b$^+$ macrophages in these mice. Similarly, there was a significantly higher frequency of activated (CD69 expression) CD11b$^+$ macrophages in immunized DR4 mice compared to the immunized WT NOD mice (Fig. 2f, $n = 4$–8) and the frequency of activated CD11b$^+$ macrophages in naive DR4 mice was also higher than their naive WT counterparts (Fig. 2f, $n = 4$–8).

DR4 mice had more inflammatory cytokine-producing T cells in the liver

To determine whether there are any functional changes in liver immune cells in response to CYP2D6/FTCD immunization, we examined the cytokine profile of the hepatic immune cells. We found that there was an increased frequency in Th1 cytokines [tumour necrosis factor (TNF)-α and interferon (IFN)-γ], Th17 cytokine interleukin (IL)-17 (Supporting information, Fig. S2a) and anti-inflammatory IL-10 (Supporting information, Fig. S2b) cytokines produced by CD4$^+$ and/or CD8$^+$ T cells after CYP2D6/FTCD immunization (Fig. 3a–e, $n = 4$–8/group). There was no difference in the frequency of these cytokine-producing T cells in naive mice (both DR4 and NOD), although a moderate increase of IL-10-producing CD4$^+$ T cells in naive DR4 mice (Fig. 3f, $n = 4$–8/group). Parallel to the cytokine profile of T cells, IL-6-producing macrophages (CD11b$^+$) and dendritic cells (CD11c$^+$) from CYP2D6/FTCD-immunized DR4 mice were also more prevalent compared to CYP2D6/FTCD-immunized WT NOD mice (Fig. 3g, $n = 4$–8/group).

Reduced liver T_{reg} frequency in immunized DR4 mice

Next, we analysed the effect of HLA-DR4 expression and/or CYP2D6/FTCD immunization on the distribution of T_{regs} (CD4$^+$FoxP3$^+$). There was no difference in the frequency of splenic T_{regs} in all groups; however, the total number of the T_{regs} was reduced in immunized DR4 mice (Fig. 4a, $n = 4$–6/group). Interestingly, the opposite pattern was seen in liver T_{regs}, which had reduced frequency in immunized DR4 mice (Fig. 4b), but there was no difference in the total number of liver T_{regs} among all the groups (Fig. 4b).

T_{regs} from naive DR4 mice have reduced inhibitory function

To test the function of T_{regs}, we first investigated the expression of PD-1, an immunoregulatory receptor that binds to its ligand PD-L1 to exert inhibitory function [30], on T_{regs} from the spleen and liver. There was a consistent reduction of PD-1$^+$ T_{regs} in spleen and liver of naive DR4 mice (Fig. 5a,b, $n = 4$–6/group); this was also the case in the liver of immunized DR4 mice, although not statistically significant (Fig. 5a,b). Next, we tested T_{reg} function. Due to the reduced viability and insufficient number of hepatic T_{regs}, we tested splenic T_{regs} for the inhibitory function in a MLR assay. We co-cultured the splenocytes from naive WT NOD mice (as responder, also the target cells for suppression) with irradiated C57BL/6 splenocytes (as an alloantigen) in the presence or absence of splenic T_{regs} from naive WT NOD or DR4 mice. The results were presented as the percentage of inhibition to the responder cell proliferation ([^{3}H]-thymidine incorporation), which was the counts per minute (cpm) in the presence of T_{regs}/cpm in the absence of T_{regs}. As shown in Fig. 5c, compared to naive WT NOD T_{regs} the T_{regs} from naive DR4 mice showed impaired suppressive function ($n = 3$–4/group). The impaired suppression is in line with the aforementioned reduction of PD-1 expression by these cells.

Increased immune response to liver autoantigen in DR4 mice *in vitro*

To investigate the autoantigen-specific immune response, we performed proliferation assays using splenocytes from CYP2D6/FTCD-immunized DR4 and NOD mice. Consistent with the hepatic inflammation seen in immunized DR4 mice, splenocytes from these mice also showed a higher proliferative response to the liver autoantigen and to pan-T cell stimulation by anti-CD3 (clone 2C11) in comparison to immunized WT NOD mice (Fig. 5d, $n = 3$/group), although the difference was not statistically significant.

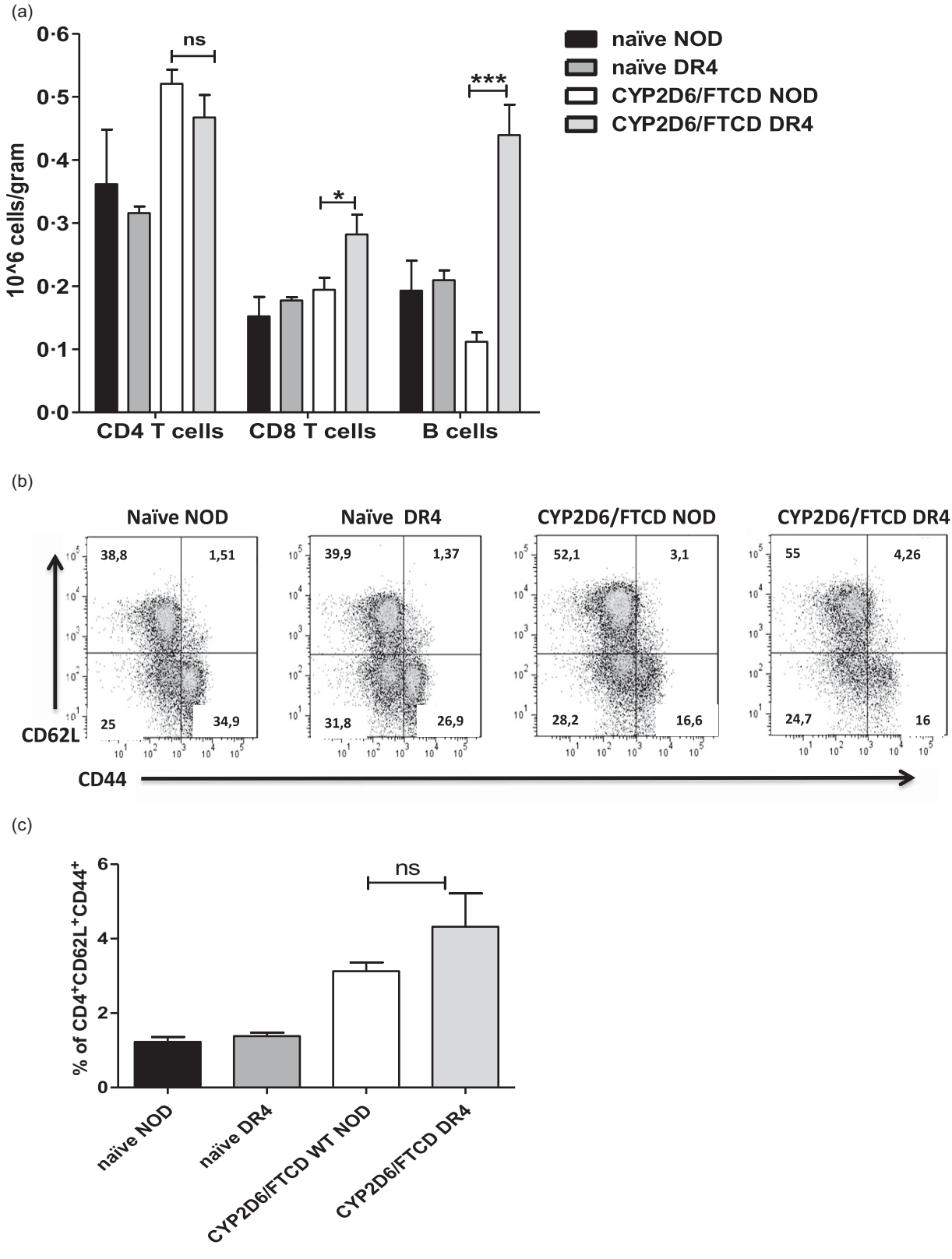

Fig. 2. Continued

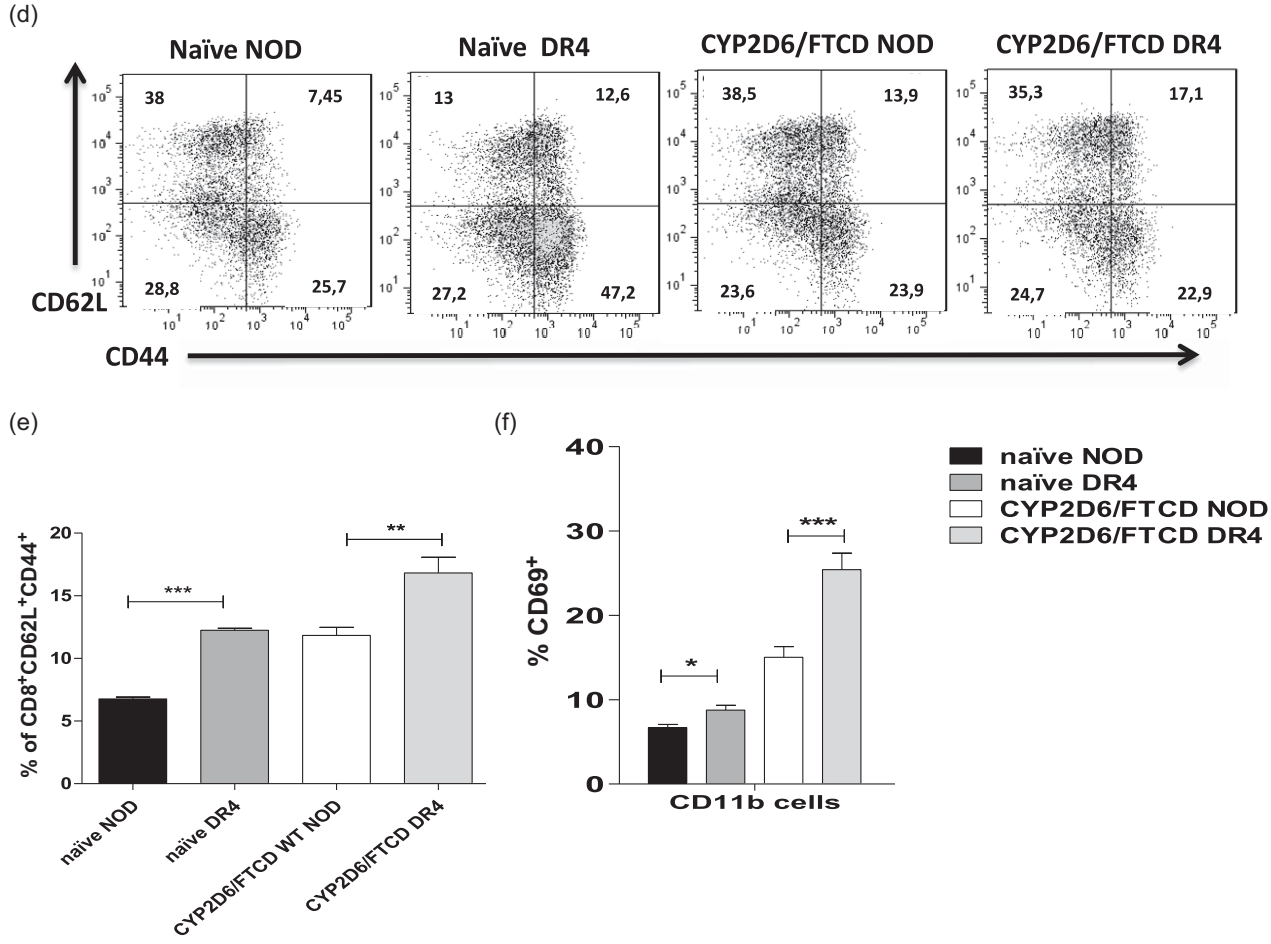

Fig. 2. Flow cytometry analysis of hepatic immune cell composition. Liver mononuclear cells (LMNCs) were isolated from DR4 or wild-type (WT) non-obese diabetic (NOD) mice 4 months after immunization and from the age-matched naive DR4 and WT NOD mice. LMNCs were stained with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD19, anti-CD11b, anti-CD69, anti-CD44 and anti-CD62L. (a) Summary of immune cell composition of LMNCs in naïve and CYP2D6/FTCD-immunized mice ($n = 4$–8/group). (b) Representative fluorescence activated cell sorter (FACS) plot of hepatic immune cells after gating on $CD4^+$ T cells. (c) Summary of the percentage of effector hepatic $CD4^+$ T cells ($n = 4$–8/group). (d) Representative FACS plot of hepatic immune cells after gating on $CD8^+$ T cells. (e) Summary of the percentage of effector hepatic CD8 T cells ($n = 4$–8/group). (f) Summary of the percentage of activated hepatic macrophages after gating on $CD11b^+$ cells. The data presented in the figures are from two pooled independent experiments and the error bars represent the standard error of the mean (s.e.m.) of samples within a group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; n.s. = not significant.

Discussion

In this study, we investigated the role of HLA-DR4 in a liver autoantigen-induced AIH in transgenic NOD mice expressing human HLA-DR4. Our major findings were: (1) that HLA-DR4 confers AIH susceptibility; (2) some features of the induced AIH in DR4 NOD mice appear to be similar to human AIH, in particular a fluctuant disease course after induction of liver damage; (3) the severity of AIH seen in DR4 mice is not as striking as we reported recently in DR3 mice, which may support the notion that DR4 is a secondary AIH susceptibility HLA allele; (4) the expression of DR4 results in more activated hepatic T cells, macrophages and dendritic cells, which may facilitate autoantibody production (anti-LKM1/anti-LC1); however, the expression of DR4 alone is not sufficient for developing spontaneous (autoimmune) liver damage; and (5) the expression of DR4 also results in impaired T_{reg} function that may explain the enhanced immune cell activation status and the susceptibility to AIH development upon autoantigen immunization.

It is interesting that the liver injury in CYP2D6/FTCD-immunized DR4 mice, when assessed biochemically, demonstrates a fluctuant pattern of transaminase levels, which is typical for human AIH. It should be noted that this particular ALT course, in DR4 as well as in NOD mice, is different from the unremitting and progressively increasing ALT levels observed in the HLA-DR3 transgenic NOD mice [16]. One possible explanation for this phenomenon is that

this difference could be the result of a different immunization protocol used in the HLA-DR3 model, which had triple immunization, whereas in the current study DR4 mice were immunized twice. Nonetheless, the relapsing pattern of ALT in DR4 mice indicates that the DR4 molecule facilitates the induction of AIH. This notion was supported by the presence of other typical features of AIH in immunized DR4 mice, such as hypergammaglobulinaemia, portal inflammation, interface hepatitis and mild fibrosis. In addition, this phenomenon could be a true reflection of what occurs in human AIH, in which less severe AIH is seen in HLA-DR4-positive patients, especially in children [17,18]. Compared to HLA-DR3-positive AIH patients, those with an HLA-DR4 allele were diagnosed at an older age, relapsed less frequently and, most importantly, had less severe disease activity. The similar findings between the human studies and our HLA-DR4 mouse model support the notion that the HLA-DR4 allele is indeed an independent risk gene which results in a milder form of AIH in both young children and old adults. It should also be noted that besides the HLA-DR4 gene, the NOD genetic background probably contributes to the liver inflammatory phenotype in our mouse model for AIH. It is well acknowledged that the NOD mouse expresses several autoimmune susceptibility genes [19–21], and this is consistent with the finding that non-major histocompatibility complex (MHC) genes have been shown to be associated with AIH in humans. Therefore, it is conceivable that these other genes in NOD mice also contribute to the development of AIH. A similar phenomenon has been seen in other types of autoimmune liver diseases, including a primary biliary cirrhosis (PBC)-like pathology mediated by B lymphocytes [31] and autoimmune cholangitis [32–34]. DR4 transgenic mice have been used for other autoimmune disease models, such as type 1 diabetes [35–37] and rheumatoid arthritis [38], although not on a NOD genetic background.

In this study, we showed autoantigen-specific T cell response after immunization. This suggests that the liver damage is mediated most probably by the autoreactive T cells in DR4 mice. We also demonstrated a high number of total B cells and plasma B cell subsets in the inflamed liver, which is similar to human AIH. Importantly, we found that in CYP2D6/FTCD-immunized DR4 mice, there were more hepatic-infiltrated T cells, both $CD4^+$ and $CD8^+$, secreting proinflammatory TNF-α, IFN-γ and IL-17 than that in CYP2D6/FTCD-immunized WT NOD mice. It is interesting that there were also more liver $CD4^+$ T cells in immunized DR4 mice secreting the anti-inflammatory cytokine IL-10. This might be explained by the fact that the regulatory mechanism (here IL-10 production) is increased to counterbalance the proinflammatory cytokine production in AIH development. In fact, we found that the vast majority of IL-17- or IFN-γ-producing CD4 T cells did not produce IL-10 (data not shown). This implies that higher levels of IL-10 are due to an increase of particular CD4 T cell subsets producing specifically IL-10, such as Tr1. A similar trend was observed in naive DR4 mice, which suggests that the HLA-DR4 molecule may alter the profile of cytokine production by immune cells intrinsically. This effect could be enhanced further by macrophages and dendritic cells that produce more IL-6. It is known that IL-6 can activate T cells, inducing chronic inflammation [39]. In addition, IL-6 promotes B cells to produce IgG [40]. This could explain that livers from CYP2D6/FTCD-immunized DR4 mice had the highest number of plasma cells, an increased number of $B220^+CD19^+$ B cells and hypergammaglobulinaemia, all of which are the hallmarks of human AIH.

The capacity of B cells to produce liver autoantibodies seems comparable in both CYP2D6/FTCD-immunized DR4 mice and WT NOD mice as all being positive for anti-LKM1/anti-LC1 autoantibodies. It is noteworthy that the titre of anti-LKM1/anti-LC1 seen in our model was very similar to a different mouse model reported by Lapierre *et al.* [7]; both were tested at 4 months after CYP2D6/FTCD immunization. As discussed earlier, it is not surprising that naive NOD mice were positive for autoantibodies, as mice of the NOD background carry several genes known to predispose to autoimmunity. This may have triggered the spontaneous autoantibody production in these mice. However, it remains unclear why the majority of naive DR4 NOD mice did not also produce anti-LKM1/anti-LC1 autoantibodies. Although CYP2D6/FTCD-immunized DR4 mice had more IgG and an increased frequency and number of plasma cells, these mice did not present with higher titres of autoantibodies compared to their WT counterparts. It seems that HLA-DR4 affects the capability of plasma cells to produce anti-LKM1/anti-LC1 autoantibodies, indicating that HLA-DR4 genes may have a protective role in the process of AIH induction. Therefore, our finding further confirms that the experimental AIH in our mouse model is mainly an immune cell-mediated disease, which mirrors what has been observed in human AIH [41].

We and others have described that impairment of $CD4^+CD25^{high}FoxP3^+T_{regs}$ contributes to the perpetuation of autoimmune responses in AIH patients [42–46]. Nonetheless, T_{reg} impairment in AIH is still a matter of debate [47]. Similar to what we found in the HLA-DR3 mouse model, DR4 mice also showed reduced T_{regs}, either the frequency or the absolute number in the liver and spleen, especially in the mice with AIH induced by autoantigen immunization. More importantly, the function of T_{regs} from DR4 mice shows an impaired inhibitory capacity compared to the T_{regs} from WT NOD mice. The PD-1/PD-L1 pathway has been well defined as a negative regulatory system, with co-expression of PD-1 on T_{regs} known to be associated with their suppressive function [48]. We found that the frequency of PD-1-expressing hepatic T_{regs} was much lower in both naive and immunized DR4 mice than in the control WT NOD mice. The splenic T_{regs} also showed reduced

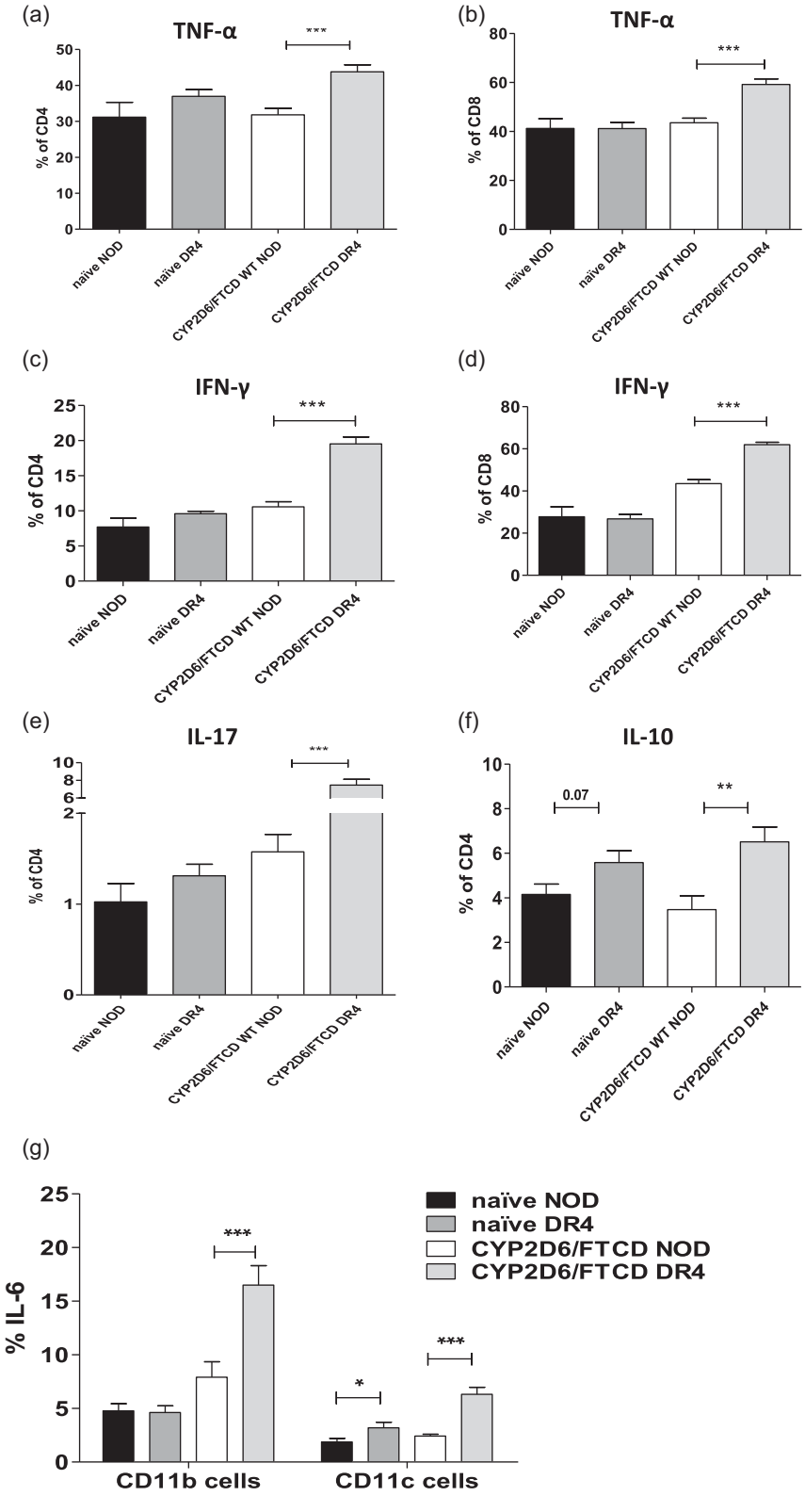

Fig. 3. Cytokine profile of liver mononuclear cells (LMNCs) in human leucocyte antigen (HLA)-DR4 mice after immunization. *Ex-vivo* LMNCs of immunized and naive mice were stained for intracellular cytokines and different surface markers as described in Materials and methods. (a–e) Summary of percentages of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-17, IL-10-producing $CD4^+$ and/or $CD8^+$ cells, and IL-6-producing $CD11b^+$ and $CD11c^+$ cells ($n = 4$–8/group). Error bars represent the standard error of the mean (s.e.m.) of samples within a group. The data presented are from two independent experiments $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

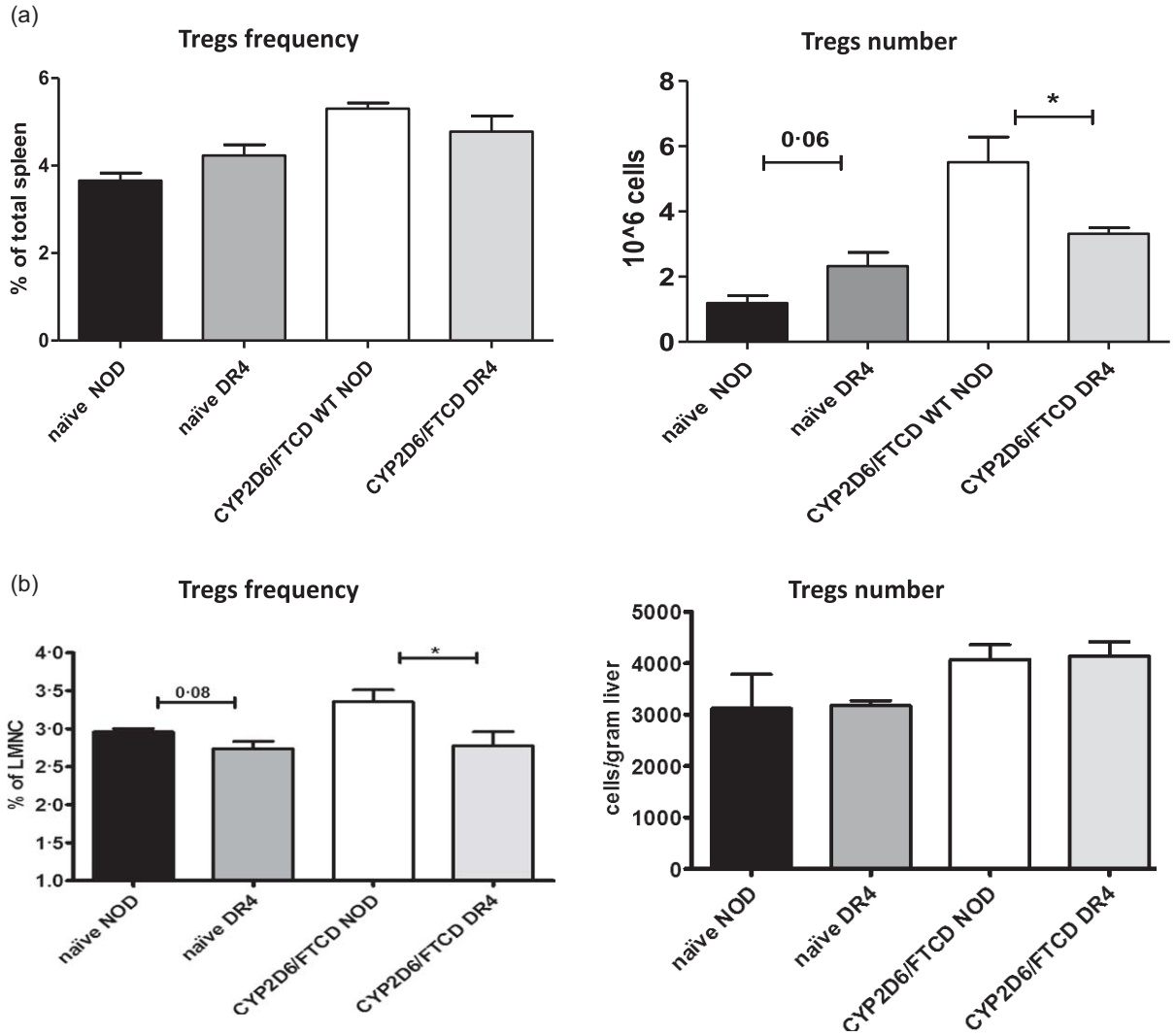

Fig. 4. Regulatory T cell (T_{reg}) populations in spleen and liver. Summary of percentages and total numbers of T_{regs} in the spleen (a) and liver (b) ($n = 4$–6/group). Error bars represent the standard error of the mean (s.e.m.) of samples within a group. The data presented are from two to three pooled independent experiments. $^*P < 0{\cdot}05$.

expression of PD-1 in DR4 mice. The reduction of PD-1 expressing T_{regs} may explain, at least in part, the impaired function of peripheral T_{regs} in DR4 mice. In addition to PD-1, deficiencies in the other member of the CD28/CD80 superfamily, the cytotoxic T lymphocyte antigen 4 (CTLA-4), might also be involved in T_{reg} impairment [49]. It is clear that this needs further mechanistic studies.

It is noted that the adjuvant CpG ODN alone caused elevation of ALT in the DR4 mice; however, the liver pathology and immunological profiles do not support the notion that the adjuvant alone can induce AIH.

In summary, we have demonstrated that HLA-DR4 plays an important role in AIH development through different pathways. This model is complementary to our DR3 model reported recently [16]. In view of the fact that broad and lifelong non-specific immunosuppressive medication is used in treating patients with AIH, resulting in severe (corticosteroid-related) side-effects [50], our mouse model enables us (i) to investigate the mechanisms of HLA-DR4 mediated immune cell activation in the lymphoid tissue and in the liver and (ii) to identify the pathway(s) involved in T_{reg} impairment. We hope that these investigations will lead ultimately to the development of novel immunotherapies to treat AIH. Takana and colleagues have shown recently that CTLA4-Ig can alleviate the ongoing autoimmunity in a mouse model for primary biliary cirrhosis [32]. More relevant to the current study is that HLA-DR4 molecules play an important role in developing autoantigen-specific immunotherapy, demonstrated by Gibson *et al.* that immunization with autoantigen alone or with autoantigen-loaded tolerogenic DCs can induce antigen-specific tolerance in HLA-DR4 Tg mice for type 1

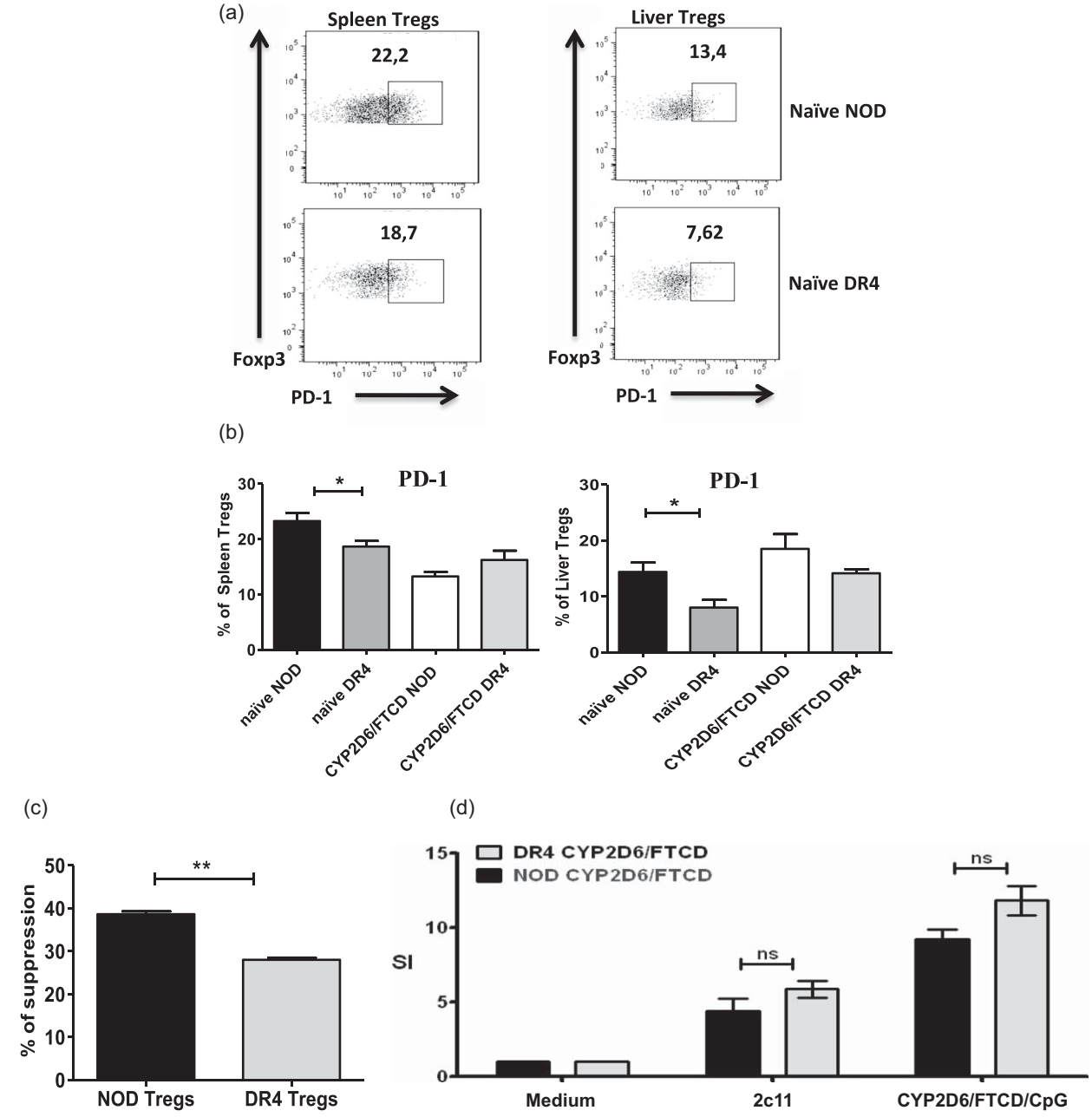

Fig. 5. Phenotype and function of regulatory T cells (T_{regs}). (a) Representative fluorescence activated cell sorter (FACS) plot of T_{regs} [$CD4^+$forkhead box protein 3 $(FoxP3)^+$ T_{regs}] expressing programmed death 1 (PD-1). (b) Summary of percentages of PD-1 expression by splenic and hepatic T_{regs}. Immune cells were stained with fluorochrome conjugated anti-CD4, anti-CD8, anti-PD1 and FoxP3 ($n = 4$–6/group). (c) T_{reg} suppression assay. T_{reg} suppression assay was performed as described in the Materials and methods. Summary of the percentage of suppression of alloreactivity in the presence of DR4 or WT, NOD splenic T_{regs} ($n = 3$–4/group). (d) T cell response to anti-CD3 stimulation or liver autoantigen CYP2D6/FTCD. Stimulation index (SI) is calculated by T cell proliferation ($[^3H]$-thymidine incorporation) in the presence of anti-CD3 or liver autoantigen/T cell proliferation ($[^3H]$-thymidine incorporation) in the absence of anti-CD3 or liver autoantigen. Medium (baseline control) is T cell proliferation in the medium only ($n = 3$/group). Error bars represent the standard error of the mean (s.e.m.) of samples within a group. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; ns = not significant.

diabetes (T1D) [37]. More recently, it has been shown by Clemente-Casares and colleagues that disease-specific autoantigens can be administered as complexes in which antigenic peptides bound to MHC class II molecules (pMHC-II) are coated on nanoparticles. Only the systemic application of nanoparticles coated with pMHC-II complexes, but not peptides or uncoated nanoparticles alone, trigger the conversion of T effector to type 1-like $CD4^+$ T and expansion of regulatory B cells. These cells ease disease severity in paralyzed experimental autoimmune encephalitis in HLA-DR4 transgenic C57BL/6 mice, and reduce the joint swelling and cartilage/bone destruction of experimental arthritis in HLA-DR4 transgenic C57BL/10 mice [51]. It is clear that antigen-specific immunotherapy may hold the promise for treating autoimmune diseases.

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Author contributions

MY performed most of the experiments and wrote the manuscript, XX performed most of the experiments and wrote the manuscript, NT performed the experiments, FA designed and supervised the CYP2D6/FTCD generation, ZH, IC and YM supervised the study and edited the manuscript, LW conceived and supervised the study and wrote the manuscript.

Disclosures

The authors have no conflict to disclose.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Liver fibrosis. (a) Fibrosis within the liver was scored (METAVIR) by an investigator unaware of the experimental groups. The data are pooled from two to three experiments ($n = 6$/group). (b) Sirius Red staining showed some periportal fibrosis in CYP2D6/FTCD-immunized DR4 and non-obese diabetic (NOD) mice ($n = 6$/group).

Fig. S2. Interleukin (IL)-17- and IL-10-producing CD4 T cells in wild-type (WT) and human leucocyte antigen (HLA)-DR4 mice. Representative fluorescence activated cell sorter (FACS) dot-plots of IL-17 (a)- and IL-10 (b)-producing CD4 T cells in naive and CYP2D6/FTCD-immunized WT and DR4 NOD mice.